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Microphthalmia-Associated Transcription Factor as a Regulator for Melanocyte-Specific Transcription of the Human Tyrosinase Gene

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Tyrosinase is a rate-limiting enzyme in melanin biosynthesis and is specifically expressed in differentiated melanocytes. We have identified the enhancer element in the 5′-flanking region of the human tyrosinase gene that is responsible for its pigment cell-specific transcription and have termed it tyrosinase distal element (TDE) (positions −1861 to −1842). Transient expression assays showed that TDE confers efficient expression of a firefly luciferase reporter gene linked to the tyrosinase gene promoter in MeWo pigmented melanoma cells but not in HeLa cells, which do not express tyrosinase. TDE was specifically bound by nuclear proteins of MeWo and HeLa cells, the binding properties of which were indistinguishable in gel mobility shift assays. TDE contains the CATGTG motif in its center, and mutation analysis indicates that the CA dinucleotides of this motif are crucial for protein binding and pigment cell-specific enhancer function. The CATGTG motif is consistent with the consensus sequence recognized by a large family of transcription factors with a basic helix-loop-helix structure, which prompted us to examine the possible involvement of a ubiquitous transcription factor, USF, and a novel factor, microphthalmia-associated transcription factor (MITF), recently cloned as the human homolog of the mouse microphthalmia (*mi*) gene product. The *mi* phenotype is associated with a mutant *mi* locus and characterized by small eyes and loss of melanin pigments. Both USF and MITF are predicted to contain a basic helix-loop-helix structure and a leucine zipper structure. We provide evidence that USF binds to TDE, whereas we were unable to detect the DNA-binding activity of MITF. Transient coexpression assays showed that MITF specifically transactivates the promoter activity of the tyrosinase gene through the CATGTG motif of TDE but not the promoter of the ubiquitously expressed heme oxygenase gene, while USF is able to activate both promoters. These results indicate that MITF is a cell-type-specific factor that is capable of activating transcription of the tyrosinase gene.

Melanin, a principal pigment found in mammals, plays an essential role in the protection against damage caused by UV light, particularly in humans. Melanin production is specifically seen in the differentiated melanocytes that originate from the neural crest or in the retinal pigmented epithelium derived from the optic cup of the brain. Tyrosinase (EC 1.14.18.1) is a rate-limiting enzyme in melanin biosynthesis that catalyzes the conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), DOPA to DOPAquinone, and possibly 5,6-dihydroxyindole to indole-5,6-quinone (14). Recent studies have shown that two other enzymes are also involved in melanin production: tyrosinase-related protein-1 (TRP-1) (18, 42) and tyrosinase-related protein-2 (TRP-2) (19, 51). TRP-1 was reported to catalyze the conversion of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) to indole-quinone-carboxylic acid (20), and TRP-2 possesses the activity of DOPAchrome tautomerase (EC 5.3.2.3), catalyzing the conversion of DOPAchrome to DHICA (54). There is about 40% amino acid identity among human tyrosinase (1, 24, 43), TRP-1 (7, 52), and TRP-2 (3, 54), all of which constitute the tyrosinase protein family and are specifically expressed in melanin-producing cells.

Both mouse and human tyrosinase genes have been characterized as models to study the regulation of cell-specific and stage-specific transcription. The promoter function of the mouse tyrosinase gene in transgenic mice was analyzed, showing that its 5′-flanking region of only 270 bp is sufficient to direct its expression specifically in skin melanocytes and retinal pigment cells of the transgenic mice (23). This 270-bp segment is also sufficient to confer cell-specific expression in cultured human melanoma cells (44). Yet, the minimum and essential element that confers pigment cell-specific expression was not determined. We have been working on the regulation of melanin production in humans and have clarified the molecular basis of tyrosinase-negative oculocutaneous albinism, which is caused by a mutation in the tyrosinase gene (48, 50). More recently, we have identified the 39-bp *cis*-acting element in the 5′-flanking region of the human tyrosinase gene that is responsible for its pigment cell-specific expression (44), namely, an element located about 1.8 kb upstream from the transcription initiation site that enhances the transient expression of the reporter gene specifically in melanoma cells.

In this study, we have determined the nucleotide sequence of the 5′-flanking region of the human tyrosinase gene and identified the exact position of the 39-bp enhancer element responsible for its pigment cell-specific transcription, which was then narrowed to the 20-bp sequence, termed the tyrosinase distal element (TDE) (positions −1861 to −1842). TDE, containing a CATGTG motif in its center, was specifically bound by nuclear proteins which are present not only in melanoma cells but also in HeLa cells, which do not express the tyrosinase gene. Mutation analysis established the significance of a CANNTG motif in protein binding and pigment cell-specific enhancer function. Furthermore, we identified the

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additional *cis*-acting element (positions −112 to −93), tyrosinase proximal element (TPE), containing a CATGTG motif, that may be required for efficient expression of the human tyrosinase gene in pigment cells.

Various types of transcription factors that control gene expression during development and differentiation have been identified and are classified according to the type of their DNA binding. A CANNTG motif is the consensus sequence of the binding site for a large family of transcription factors with a basic helix-loop-helix (bHLH) structure (29, 30). The bHLH structure is required for DNA binding and dimerization of transcription factors. A ubiquitous transcription factor, upstream stimulatory factor (USF), is one such factor containing a bHLH structure and a leucine zipper (LZ) structure (31). USF was initially identified as a nuclear protein that binds to the upstream element of the adenovirus major late promoter (38) and then shown to be involved in the expression of various cellular genes (5, 6, 25, 34, 37, 39). More recently, USF was shown to bind to the mouse TRP-1 gene promoter (53). Furthermore, a novel factor with a bHLH-LZ domain was recently cloned from the mouse microphthalmia (*mi*) locus (15, 16, 46). The *mi* phenotype is associated with the mutant *mi* locus, and mice with this phenotype are characterized by small nonpigmented eyes and a lack of melanocytes in the skin and inner ear. The human homolog of the mouse *mi* gene product was then cloned and termed microphthalmia-associated transcription factor (MITF) (47). MITF is closely related to the bHLH-LZ family of transcription factors, which prompted us to examine the possible involvement of MITF in melanocyte-specific transcription of the tyrosinase gene. Here, we provide evidence that USF is one of the TDE- and TPE-binding proteins and is involved in the efficient transcription of the tyrosinase gene in melanocytes. In addition, we found that MITF activates the pigment cell-specific promoter function of the tyrosinase gene through TDE. Because the disruption of the *mi* locus by a transgenic insertional mutation led to the *mi* phenotype (15, 46) and the *mi* gene was reported to be expressed in melanoblasts, which are precursors to melanocytes (15), the *mi* gene product (Mi protein) or its human homolog, MITF, may play an essential role in the differentiation of neural crest cells toward melanocytes and in the maintenance of the differentiated phenotype of melanocytes.

MATERIALS AND METHODS

DNA sequencing. The *Xba*I-*Pst*I fragment containing the 2.7-kb 5′-flanking region of the human tyrosinase gene (44, 48) was subjected to sequence analysis. Briefly, the fragment was digested with the appropriate restriction enzymes and the resulting smaller fragments were subcloned into the cloning vector pBluescript II (Stratagene) and sequenced by 373A Autosequencer (Applied Biosystems Inc.). All DNA fragments were sequenced in both directions.

Plasmid construction. A promoterless plasmid, pL1, and the fusion plasmids containing the human tyrosinase promoter, pHTL6, pHTL10, pHTL12, and pHTL17 (all of which contain the firefly luciferase gene as a reporter gene), were described previously (44). The plasmids pHTL18, pHTL28, pHTL29, and pHTL21 contain the fragments generated by digesting the *Eco*RI-*Nco*I fragment (positions −2014 to −1810) with either *Hin*fI or *Mse*I at the 5′ end of the tyrosinase promoter (position −209) of pHTL10 (see Fig. 2A). For insertion of the synthetic oligonucleotides containing a *Sal*I or *Xho*I site at each 5′ end, pHTL10 was modified by inserting an *Sal*I linker at the 5′ end of its tyrosinase promoter (position −209), which was designated pHTL10S. The oligonucleotides were inserted at the *Sal*I site of pHTL10S (see Fig. 4B).

The fusion plasmids used for functional analysis of the proximal promoter region were constructed as follows. pHTL6 (44) linearized with *Bam*HI was digested with either *Ase*I (position −184), *Stu*I (position −150), *Bst*BI (position −120), or *Hin*dIII (position −82), and the ends were filled in with Klenow fragment. Each larger fragment was self-ligated with T4 DNA ligase, yielding pHTL25, pHTL26, pHTL27, and pHTL8 (see Fig. 5A). All constructs used in this study were confirmed by sequencing with the dideoxy chain termination method (36). The construct pHHOSVL4 contains the *Msc*I-*Xho*I fragment (positions −176 to +19) of the human heme oxygenase gene (40) upstream from the luciferase gene.

Cell culture and transient expression analysis. MeWo human melanoma cells (2) were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 7% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. HeLa human cervical cancer cells were cultured in minimum essential medium under the same conditions. HMV-II human melanotic melanoma cells (13, 17) were obtained from RIKEN Cell Bank (Tsukuba, Japan) and cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

For DNA transfection, 8×10^5 MeWo cells or 5×10^5 HeLa cells were seeded in a 6-cm-diameter dish 1 day before the procedure. Transfection was performed by the calcium phosphate precipitation method (11) as described previously (45). The amounts of DNA used for cotransfection were 7 μg of a test plasmid and 1.4 μg of pCH110, a β-galactosidase expression vector containing the simian virus 40 (SV40) early promoter as an internal control (Pharmacia LKB Biotechnology). After 24 h, transfected cells were harvested and lysed with 0.1 M potassium phosphate buffer (pH 7.4) containing 1% Triton X-100. Soluble extracts were then assayed for luciferase activity (8) and β-galactosidase activity (35) as an internal control. Luciferase activity was normalized by β-galactosidase activity, which was then divided by the value obtained with pSV2/L (relative luciferase activity). pSV2/L, containing the luciferase gene under the SV40 early promoter (8), was kindly provided by S. Subramani (University of California at San Diego, La Jolla).

Preparation of nuclear extracts and fractionation on a phosphocellulose column. Nuclear extracts were prepared by to the method of Dignam et al. (9) and stored at −80°C. Protein concentrations were measured as described by Bradford (4). Crude nuclear extracts were applied to a column filled with P-11 phosphocellulose resin (Whatman), previously equilibrated with buffer D containing 100 mM KCl (9). After the column was washed extensively with buffer D containing 200 mM KCl, bound proteins were eluted with buffer D containing 320 mM KCl. Each fraction was subjected to a gel mobility shift assay. Fractions containing the TDE-binding activity were collected and dialyzed against buffer D containing 100 mM KCl.

Gel mobility shift assay. A synthetic oligonucleotide was end-labeled with [γ-^{32}P]ATP (NEN Research) (specific radioactivity, 1×10^8 to 2×10^8 cpm/μg). Nuclear extracts were preincubated on ice for 20 min with poly(dI-dC) in the presence or absence of competitor DNAs. An end-labeled probe (2×10^4 cpm) was added to the preincubation mixture, and the binding reaction was performed at 24°C for 20 min. The binding mixture contained 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–NaOH (pH 7.9), 12% glycerol, 60 mM KCl, 0.3 mM dithiothreitol and phenyl-

methylsulfonyl fluoride, 0.12 mM EDTA, 2 mM $MgCl_2$, 2 μg of poly(dI-dC), an end-labeled probe, and competitor DNAs. The reaction mixtures were loaded onto a 4.5% polyacrylamide gel for electrophoresis at 4°C in the running buffer containing 50 mM Tris-HCl–50 mM borate–1 mM EDTA (pH 8.0). The gel was then dried and subjected to autoradiography. In one series of experiments, the binding reaction was performed in the presence of the anti-USF antibody (31), provided by Robert G. Roeder (Rockefeller University, New York, N.Y.).

DNase I footprinting analysis. The *Eco*RI-*Nco*I fragment (positions −2014 to −1810) of the tyrosinase gene was filled in with Klenow fragment in the presence of [α-^{32}P]dCTP (NEN Research) to label only the *Nco*I end and was used as a probe for DNase I footprinting analysis. Binding reactions were performed as described above for the gel mobility shift assay but in a 25-μl reaction volume containing increasing amounts (0 to 16 μg) of fractionated MeWo nuclear extracts. Other procedures were performed as described previously (28). For a sequence ladder, the probe fragment was partially cleaved by the method of Maxam and Gilbert (27).

Cloning of human MITF cDNA. For isolation of human MITF cDNA, the mouse MITF cDNA fragments were prepared from B16 melanoma RNA by PCR using the primers 5′-AGTCACTACCAGGTGCAGAC-3′ (positions 151 to 170) as a forward primer and 5′-CTTGCTTCAGACTCTGTGGG-3′ (complementary to positions 700 to 719) as a reverse primer (15). The identity of PCR products was confirmed by sequencing. The amplified fragments were labeled with [α-^{32}P]dCTP and used as a probe to screen an HMV-II human melanoma cDNA library constructed in λgt11 (54). Nine positive phage clones were isolated, and an insert of each was prepared by digestion with *Eco*RI and cloned into the *Eco*RI site of the pBluescript vector. One of these subclones, called hMI-9, carries the 1.6-kb insert containing an entire protein-coding region of human MITF.

In vitro transcription and translation. Two plasmids, dI2 containing a USF cDNA (12) and phMI-9 containing human MITF cDNA, were linearized by digestion with *Spe*I and used as templates. Capped RNA was synthesized by using T7 RNA polymerase and P^1-5′-(7-methyl)-guanosine-P^3-5′-guanosine triphosphate (Boehringer Mannheim) as a cap analog. The reaction mixture (25 μl) contained 40 mM Tris-HCl (pH 7.9); 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl; 0.5 mM (each) ATP, CTP, and UTP; 0.05 mM GTP; 0.25 mM P^1-5′-(7-methyl)-guanosine-P^3-5′-guanosine triphosphate; 10 mM dithiothreitol; 40 U of RNasin (Promega); 1 μg of linearized template DNA; and 40 U of T7 RNA polymerase. The reaction mixtures were incubated at 39°C for 1 h. After digestion with RNase-free DNase I, the synthesized RNAs were extracted with phenol-chloroform (1:1) and precipitated with ethanol. The quality of synthesized RNAs was checked in formaldehyde-denatured agarose gel. In vitro translation of the capped RNAs was performed simultaneously in the presence or absence of L-[^{35}S]methionine (Amersham), using rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. Translated products with L-[^{35}S]methionine were visualized by autoradiography. A portion (2 μl) of the translation mixture, lacking L-[^{35}S]methionine, was used for the gel mobility shift assay.

Expression of human MITF and USF cDNAs. Both ends of the 1.6-kb *Eco*RI-*Eco*RI fragment, isolated from hMI-9, were filled in and inserted into the *Hin*dIII site of the pRc/CMV eukaryote expression vector (Invitrogen), yielding pRc/CMV-MITF. The *Hin*dIII site had been converted to blunt ends prior to ligation. The *Stu*I-*Stu*I fragment was isolated from a human USF cDNA dI2 (12), provided by Robert G. Roeder (Rockefeller University), and was similarly inserted into the *Hin*dIII site of the pRc/CMV vector, yielding pRc/CMV-USF. These constructs were transiently expressed together with a fusion gene in MeWo or HeLa cells as described above.

Nucleotide sequence accession number. The accession number of the tyrosinase gene sequence shown in this paper is D26163.

RESULTS

Nucleotide sequence of the upstream region of the human tyrosinase gene. We have identified the 39-bp element that is responsible for pigment cell-specific transcription of the human tyrosinase gene (44). To clarify the correct position of this enhancer and search for another type of potential regulatory element(s), we determined the nucleotide sequence of the 5′ upstream region of the human tyrosinase gene (Fig. 1). Our study indicates that the 39-bp enhancer element is located between positions −1874 and −1836. The 39-bp enhancer element contains a CATGTG motif in its center, a consensus sequence of the binding site for the bHLH protein (21). There are three additional copies of a CATGTG motif in the 5′ upstream region of the human tyrosinase gene (positions −1972 to −1967, −104 to −99, and −12 to −7). No consensus sequence typical of a cyclic AMP-responsive element (TGAC GTCA) or 12-*O*-tetradecanoylphorbol-13-acetate-responsive element ($TGA^C/_GT^C/_AA$) (10) is found in this sequence.

Presence of a nuclear protein that binds to the 39-bp enhancer element. The function of the 39-bp enhancer element has been established by using the fusion plasmids containing the SV40 promoter (44). Thus, to confirm the enhancer activity of this 39-bp element under the homologous promoter, we transiently expressed the fusion plasmids in MeWo melanoma and HeLa cells containing various fragments adjacent to this enhancer element at the 5′ end of the tyrosinase promoter region (position −209) of pHTL10 (Fig. 2A). Luciferase activity was preferentially expressed in MeWo melanoma cells when the fusion plasmids contained the *Mse*I-*Hin*fI fragment (the 39-bp element). In contrast, similar luciferase activity was detected in both cell lines transfected with pHTL18 lacking the *Mse*I-*Hin*fI fragment. Thus, the 39-bp element was confirmed to have the enhancer activity under the homologous promoter in a pigment cell-specific manner.

To search for a protein that binds to the 39-bp enhancer, we performed gel mobility shift assays (Fig. 2C). The oligonucleotide MH, corresponding to the *Mse*I-*Hin*fI fragment (positions −1874 to −1836), contains the 39-bp element and was used as a probe. The oligonucleotide HN (positions −1838 to −1805) includes the *Hin*fI-*Nco*I fragment adjacent to the 39-bp element. Tyrosinase element 1 (TE-1) is a putative pigment cell-specific enhancer element of the mouse tyrosinase gene proposed by Ponnazhagan and Kwon (33) and shares similarity with the 39-bp enhancer element (Fig. 2B). Specific binding activities were detected as two major retarded bands with crude MeWo nuclear extracts (Fig. 2C, lane 2). The retarded bands were not detectable when an excess amount of oligonucleotide MH was included in the binding reaction (Fig. 2C, lanes 3 and 4). In contrast, oligonucleotide HN or TE-1 exerted no effects on the formation of protein-DNA complexes (lanes 5 to 8). These results indicate that nuclear proteins in MeWo cells specifically bind to the 39-bp enhancer element but not to TE-1.

Localization of the binding region within the 39-bp enhancer element. DNase I footprint analysis was performed to determine the region required for protein binding. Because we could not detect the protected region with crude MeWo

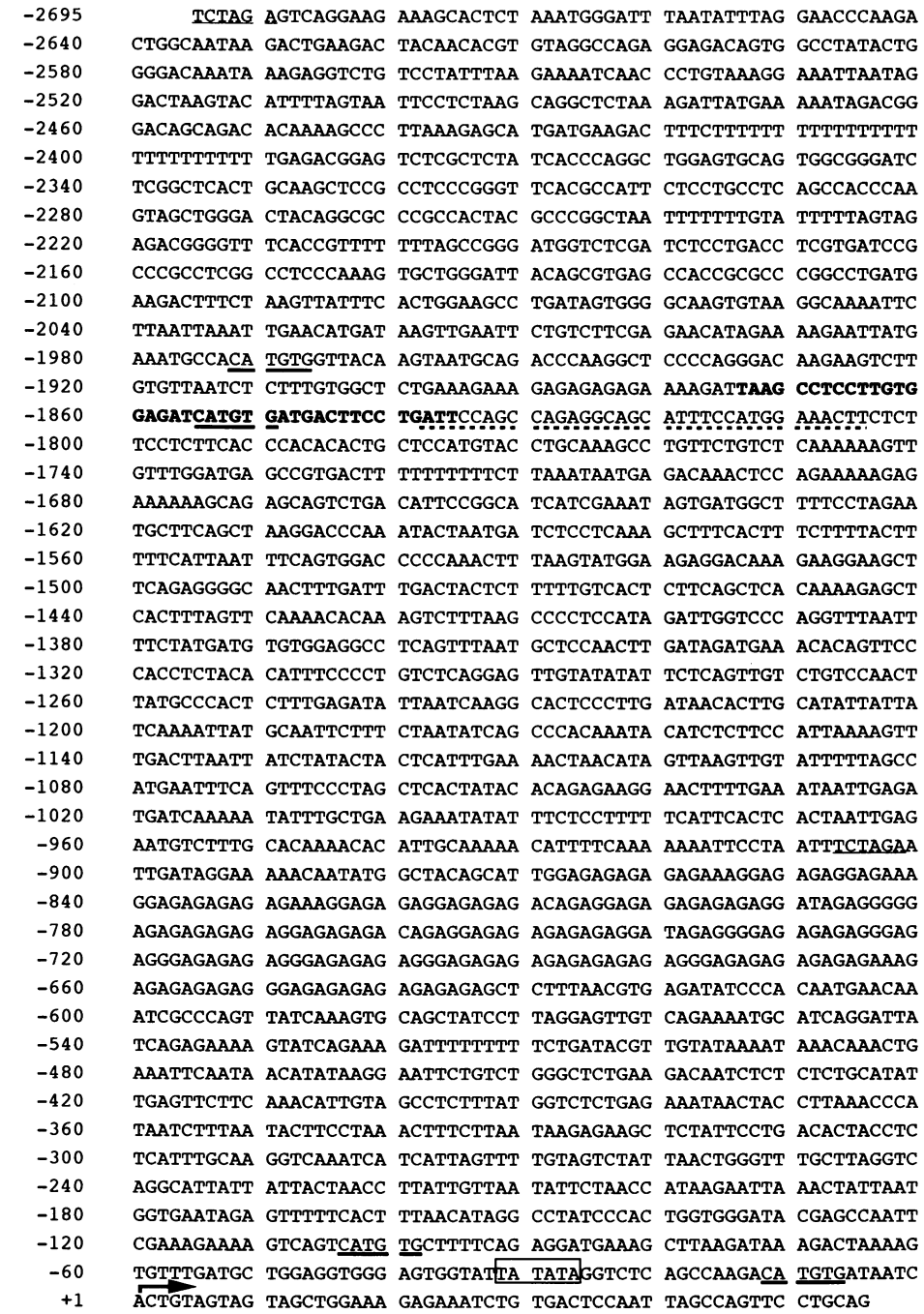

FIG. 1. Nucleotide sequence of the 5′ upstream region of the human tyrosinase gene. Only the coding-strand sequence, numbered beginning at the transcription initiation site (angled arrow), is shown. The 39-bp element responsible for pigment cell-specific expression (44) is indicated by boldface type. The region adjacent to this element, represented by oligonucleotide HN (Fig. 2A), is underlined with dashes. The deduced TATA box is in an open box. Two restriction sites for *Xba*I (thin lines) and the CATGTG motifs (thick lines) are underlined. The sequence of the 5′-flanking region downstream from the *Xba*I site at position −907 is identical to that reported by Kikuchi et al. (22). The nucleotide sequence of the 2-kb 5′-flanking region was published by Ponnazhagan et al. (32), but there are several base differences, including the deletion of the *Hin*dIII-*Hin*dIII fragment (positions −1581 to −1504 based on our numbering).

nuclear extracts, we partially purified the binding protein, using a phosphocellulose column. The binding activity was eluted in the 0.2 to 0.32 M KCl fraction (Fig. 3A) and was used for DNase I footprint analysis. The central region of the 39-bp element, GGAGATCATGTGATGACTTC (positions −1861 to −1842), was protected by partially purified protein (Fig. 3B). This 20-bp protected region was termed tyrosinase distal element (TDE) and was then subjected to further analysis.

TDE functions as a pigment cell-specific enhancer. The central position in TDE of a CATGTG motif prompted us to determine the significance of this motif. Two 20-bp oligonucleotides, TDE and TDE-M, in the latter of which CATGTG

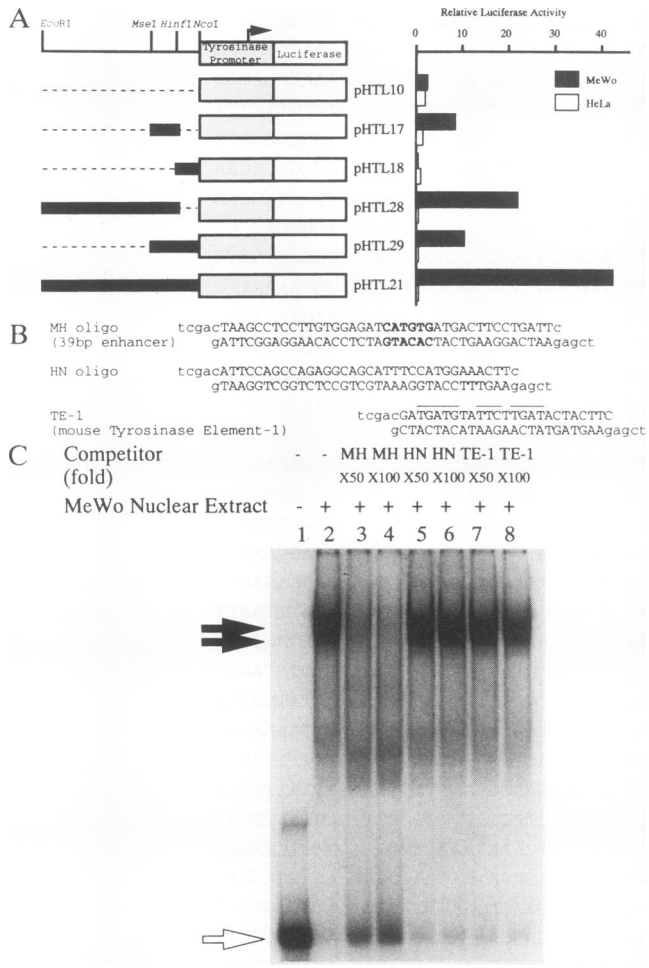

FIG. 2. Detection of nuclear proteins that bind to the 39-bp element responsible for the pigment cell-specific expression of the human tyrosinase gene. (A) Functional analysis of the 39-bp enhancer element. The fusion plasmids containing the *Eco*RI-*Nco*I fragment (positions −2014 to −1810) and its dissected fragments upstream of the tyrosinase promoter (positions −209 to +51) are schematically shown on the left. Each fusion plasmid was transiently expressed in MeWo melanoma or HeLa cells. The luciferase activities obtained were normalized by values for each β-galactosidase activity, the result of which was then divided by the value obtained with pSV2/L, shown as units of relative luciferase activity. (B) Nucleotide sequences of synthetic oligonucleotides. The oligonucleotide (oligo) MH (positions −1874 to −1836) represents the 39-bp enhancer element of the human tyrosinase gene and corresponds to the *Mse*I-*Hin*fI fragment (positions −1874 to −1838). HN oligo including the *Hin*fI-*Nco*I fragment (positions −1838 to −1805) is located at the 3′ side of the *Mse*I-*Hin*fI fragment. TE-1 (positions −240 to −216) is the possible enhancer element in the mouse tyrosinase promoter reported by Ponnazhagan and Kwon (33), and the overlines above the sequence indicate the nucleotides identical to those of oligonucleotide MH. Uppercase letters indicate the nucleotide sequence from the tyrosinase gene, and lowercase letters indicate the linker sequence used for construction. (C) Presence of proteins in MeWo nuclear extracts that bind to the 39-bp element. A gel mobility shift assay was performed with oligonucleotide MH as a probe. Two major bands are indicated by solid arrows, and unbound probes are indicated by an open arrow. All competitors were added to the reaction mixture at a 50- or 100-fold molar excess over the input probe concentration as indicated. Lane 1, no nuclear extract (buffer control); lane 2, protein-DNA complexes formed with nuclear extracts in the absence of competitor.

was modified to ACTGTG (Fig. 4A), were synthesized. Gel mobility shift assays demonstrated that TDE was bound by nuclear protein(s) present in MeWo nuclear extracts, which was again detected as two major bands (Fig. 4A, lane 2). The formation of specific DNA-protein complexes was inhibited by the oligonucleotide TDE (lanes 3 and 4), but not by TDE-M containing the ACTGTG motif (lanes 5 and 6). To confirm whether the formation of these complexes was melanoma cell-specific, we performed similar analysis using HeLa nuclear extracts. However, the formation of specific complexes was also detected as two bands with HeLa nuclear extracts (Fig. 4A, lanes 8 to 12), with patterns indistinguishable from those seen with MeWo nuclear extracts.

We then analyzed the function of TDE by transient expression of the fusion plasmids containing oligonucleotide TDE or TDE-M at the 5′ end of the tyrosinase promoter carried by pHTL10S (Fig. 4B). When the basic construct, pHTL10S, was expressed in MeWo and HeLa cells, the level of promoter activity of the tyrosinase gene was always higher in MeWo cells than that in HeLa cells but the differences were not statistically significant. When pHTL10SWT1 and pHTL10SWT3 containing TDE at the 5′ end (position −209) of pHTL10S were expressed, the relative luciferase activities were increased only in MeWo cells according to the copy number of the inserted TDE. The orientation of TDE did not affect the relative luciferase activities. It is noteworthy that the presence of three copies of TDE resulted in a remarkable increase in the relative luciferase activity only in melanoma cells but not in HeLa cells. In contrast, when pHTL10SMT containing TDE-M at the 5′ end of the tyrosinase promoter was used, no significant increases were detected (Fig. 4B). These results indicate that TDE functions as a pigment cell-specific enhancer and the CA dinucleotides of the CATGTG motif are also crucial for enhancer activity. This function of TDE and the presence of its binding protein were also confirmed in another human melanoma cell line, HMV-II (data not shown).

Functional analysis of the proximal CATGTG motifs of the human tyrosinase gene promoter. As shown in Fig. 1, there are three additional copies of the CATGTG motif in the human tyrosinase promoter region (positions −1972 to −1967, −104 to −99, and −12 to −7). It has been suggested that the CATGTG motif at positions −1972 to −1967 is not essential for pigment cell-specific expression of the tyrosinase gene (44). Consequently, we performed transient expression assays to explore the function of the two proximal CATGTG motifs (positions −104 to −99 and −12 to −7) (Fig. 5A). Higher levels of luciferase activities were detected in MeWo cells transfected with the constructs containing the two proximal CATGTG motifs (such as pHTL25, pHTL27, and pHTL10) than in HeLa cells, although the differences between MeWo and HeLa cells were not always significant. In addition, luciferase activity levels were slightly higher in MeWo cells transfected with the constructs containing the two proximal CATGTG motifs than those obtained with pHTL8 containing the CATGTG motif located at positions −12 to −7. We therefore chose the 20-bp region (positions −112 to −93), containing the CATGTG motif (positions −104 to −99), as a putative pigment cell-specific promoter and termed it the tyrosinase proximal element (TPE). The function of TPE is however not always sufficient to be detected under the conditions employed in this study. Consistent with this finding, the 1.0-kb 5′-flanking region of the human tyrosinase gene containing TPE but lacking TDE was shown to be sufficient to confer cell-specific expression of the tyrosinase gene in transgenic mice (49). It is also noteworthy that about 50 copies of the transgene containing the human tyrosinase gene promoter

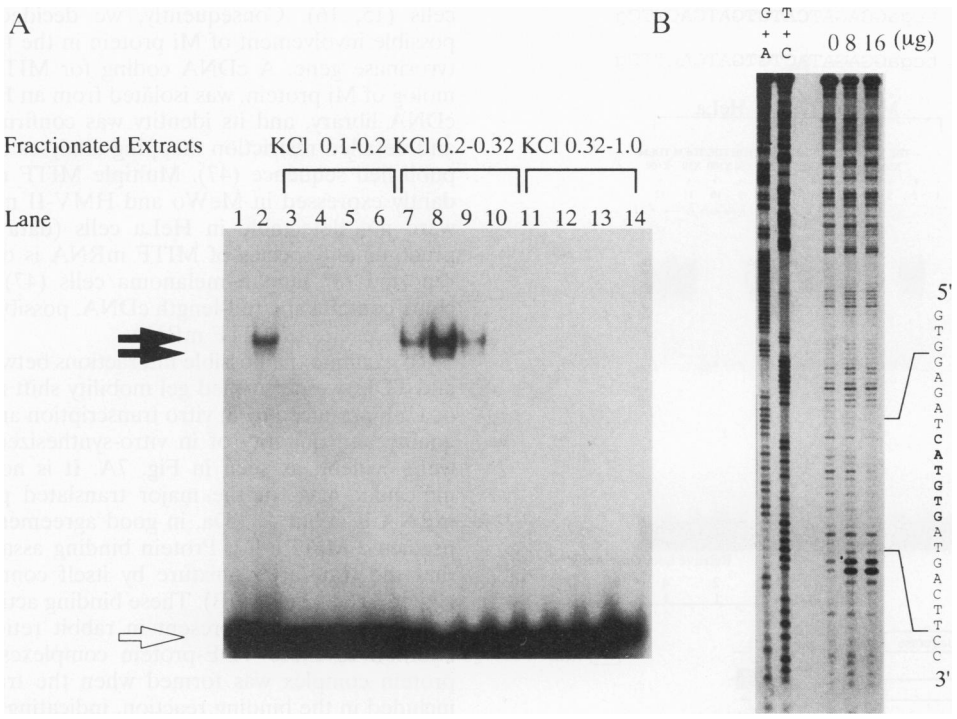

FIG. 3. Localization of the binding region of nuclear proteins within the 39-bp enhancer element. (A) Partial purification of binding proteins. MeWo nuclear extracts were applied to a P-11 phosphocellulose column equilibrated with buffer D containing 0.1 M KCl and fractionated stepwise with buffer D containing 0.2, 0.32, and 1.0 M KCl. The eluted fractions were immediately subjected to a gel mobility shift assay using the oligonucleotide MH as a probe (Fig. 2B). Lane 1, buffer control; lane 2, protein-DNA complexes formed with crude nuclear extracts. Major bands and probes are indicated as in Fig. 2C. (B) DNase I footprinting analysis. DNase I footprinting analysis was performed for the coding strand with increasing amounts of partially purified nuclear extracts as shown above the lanes. Sequence ladders were prepared by chemical cleavage of the probe DNA and are indicated as G+A (cleavage at guanine and adenine residues) and T+C (at thymine and cytidine residues).

were tandemly integrated at one site on the chromosome in the transgenic mice and that no pigmented melanocytes were detected in the epidermis (49). It is therefore conceivable that the presence of TDE is required for efficient and correct expression of the human tyrosinase gene in a pigment cell-specific manner.

Presence of nuclear proteins that bind to the proximal CATGTG motif. We then looked for a protein that binds to TPE by gel mobility shift assays using TDE as a probe (Fig. 5B). The oligonucleotide competitors used were TPE, two types of mutated TPE (TPE-M1 and TPE-M2), and TRP-1 element (TRP1E) (Fig. 5B). TPE-M1 contains a single mutation outside the CATGTG motif, and TPE-M2 (CATGTA) contains a single mutation at the 3′ end of the CATGTG motif. The sequence similar to TDE was present in the human TRP-1 gene promoter (positions −49 to −30) (45) and was termed TRP1E, although our laboratory reported earlier that the region containing TRP1E is unable to confer pigment cell-specific expression of the human TRP-1 gene (45). The formation of TDE-protein complexes was completely inhibited by TPE or TPE-M1 as well as by TDE but was not inhibited by TDE-M, TPE-M2, or TE-1 (Fig. 5B, lanes 2 to 9). These results suggest that TPE was also bound by the TDE-binding proteins and its CANNTG motif is crucial for protein binding. However, it should be noted that the formation of TDE-protein complexes was only slightly inhibited by TRP1E containing the CATGTG motif (lanes 2 and 8), suggesting that the sequence adjacent to the CATGTG motif is also required for efficient protein binding. We next compared the properties of the TPE-binding proteins with those of the TDE-binding proteins. Gel mobility shift assays using TPE as a probe revealed that two main complexes, which were indistinguishable from those formed with TDE as a probe (Fig. 5C, lanes 2 and 3), were formed with MeWo or HeLa nuclear extracts (lane 5 or 6). TPE-protein complex formation similarly involved competition by TDE, TPE, or TPE-M1 but not by TDE-M or TPE-M2 (Fig. 5C, lanes 7 to 11). Again, the magnitude of inhibition by TRP1E seemed lower than that by TPE, TPE-M1, or TDE (lane 12). Thus, the properties of the binding proteins for TDE and TPE are indistinguishable in the gel mobility shift assays.

Binding of USF to both TDE and TPE. We then analyzed the effects of synthetic oligonucleotides, representing the binding sites of well-characterized transcription factors USF, AP1, Sp1, and AP2 (Fig. 6A), on the TDE-binding activities in MeWo nuclear extracts. Only the adenovirus major late promoter, containing the USF-binding site, inhibited the formation of the specific TDE-protein complexes, and other oligonucleotides did not compete for the formation of these complexes (Fig. 6A, lanes 3 to 6), suggesting that USF binds to TDE. Immunoblot analysis showed that USF is similarly expressed in both melanoma and HeLa cells (data not shown). To obtain further evidence that the TDE-protein complexes include USF, we used the antiserum against USF in gel mobility shift assays (Fig. 6B). With MeWo nuclear extracts and TDE as a probe, addition of the antiserum resulted in the formation of a supershifted band which was not detected when phosphate-buffered saline (PBS) or preimmune serum was added to the binding reaction mixture (Fig. 6B, lanes 2 to 4). When HeLa nuclear extracts were used instead of MeWo nuclear extracts

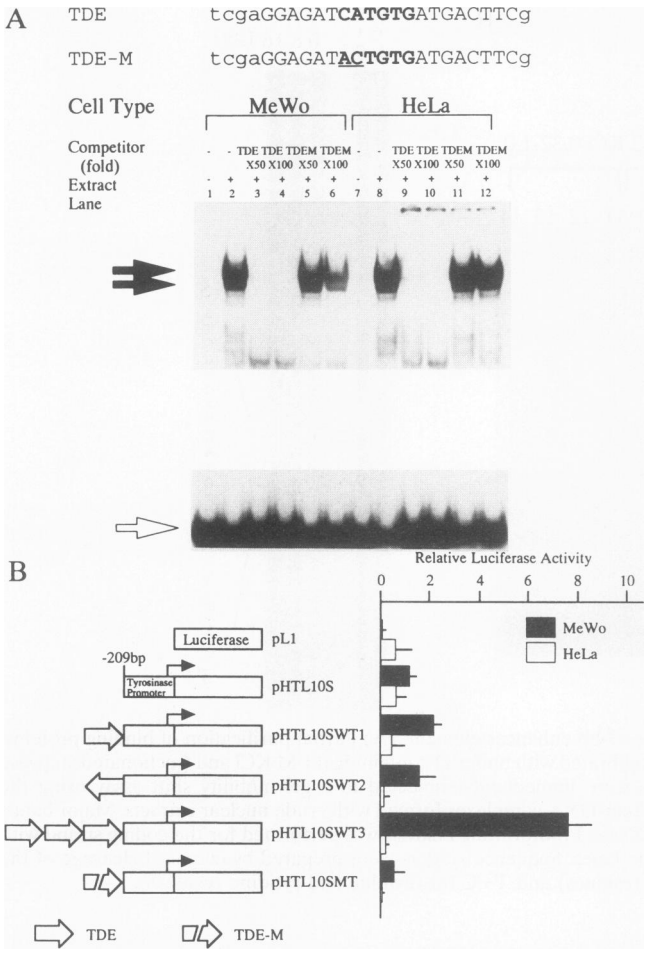

FIG. 4. Characterization of TDE. (A) Identification of nuclear proteins that specifically bind to TDE. Sequences of the synthetic oligonucleotides are indicated at the top of the figure. Only the sense strand is indicated. Uppercase letters indicate the nucleotides of the human tyrosinase gene, and lowercase letters indicate the linker sequence used for construction. TDE-M contains two base substitutions (underlined). A gel mobility shift assay was performed with the synthetic TDE as a probe. MeWo nuclear extracts (lanes 1 to 6) and HeLa nuclear extracts (lanes 7 to 12) were used. Two major complexes are indicated by solid arrows, and unbound probes are indicated by an open arrow. (B) Functional analysis of TDE. The fusion plasmids containing TDE or TDE-M upstream of the tyrosinase promoter (positions −209 to +51) are schematically shown on the left side. Other conditions are the same as those described in the Fig. 2A legend. The values shown are means ± standard deviations for three independent experiments.

(lanes 5 to 7) or TPE was used as a probe instead of TDE (lanes 9 to 14), similar results were obtained. In addition to the supershifted bands, the stacking of the sample at the origin of the lane may represent the formation of DNA-protein-antibody complexes (lanes 4, 7, 11, and 14). These results suggest that USF is a one of the TDE-binding proteins.

The possible binding of USF to the CATGTG motif in TDE and TPE reminds us of the putative transcription factor Mi protein, recently identified as an *mi* gene product (15, 16). One of the defining features of *mi* mice is a lack of melanin pigment, and Mi protein is predicted to contain a bHLH-LZ structure. In addition, expression of *mi* mRNA appears to be restricted in certain tissues and cell lines, including melanoma cells (15, 16). Consequently, we decided to examine the possible involvement of Mi protein in the transcription of the tyrosinase gene. A cDNA coding for MITF, the human homolog of Mi protein, was isolated from an HMV-II melanoma cDNA library, and its identity was confirmed by sequencing and detailed restriction mapping analyses on the basis of the published sequence (47). Multiple MITF mRNAs are abundantly expressed in MeWo and HMV-II melanoma cells but were not detectable in HeLa cells (data not shown). The predominant species of MITF mRNA is of about 5.5 kb, as reported for human melanoma cells (47), but the isolated clone contains the full-length cDNA, possibly representing the 1.8-kb species of MITF mRNA.

To examine the possible interactions between MITF or USF and TDE, we performed gel mobility shift assays using MITF or USF produced by in vitro transcription and translation. The quality and quantity of in vitro-synthesized MITF and USF were evident, as seen in Fig. 7A. It is noteworthy that the molecular mass of the major translated products of MITF mRNA is about 47 kDa, in good agreement with that of the predicted MITF (46). Protein binding assays clearly indicate that the translation mixture by itself contains TDE-binding activities 1 to 3 (Fig. 7B). These binding activities are probably due to the proteins present in rabbit reticulocyte lysate. In addition to these TDE-protein complexes, a unique TDE-protein complex was formed when the translated USF was included in the binding reaction, indicating that USF binds to TDE and the core sequence CATGTG is required for the specific protein binding (lanes 8 to 10). The mobility of the TDE-USF complex is identical to that of the upper band formed with nuclear extracts (data not shown). In contrast, we were unable to detect any specific TDE-MITF complexes (Fig. 7B, lanes 5 to 7). These results confirm the binding of USF to TDE and suggest the presence of an additional ubiquitous factor(s) binding to TDE. The DNA-binding activity of MITF remains to be investigated.

Transcriptional activation of the tyrosinase gene promoter by MITF or USF. We then analyzed the function of USF and MITF by transient cotransfection assays. As controls, we also included the SV40 early promoter and the human heme oxygenase gene promoter. The promoter region of pSV2/L contains multiple GC boxes but no CANNTG motif. Heme oxygenase is an essential enzyme in heme catabolism and is ubiquitously expressed. The human heme oxygenase gene promoter contains two functional USF-binding sites (28, 37). Thus, the SV40 promoter may function as a negative control for USF and MITF, while the heme oxygenase gene promoter possibly functions as a positive control for USF. A MITF or USF expression plasmid was coexpressed with a luciferase construct containing a promoter region of either tyrosinase, SV40, or heme oxygenase (Fig. 8). The magnitude of activation was shown as the ratio of each normalized luciferase activity to that obtained with vector DNA (shown as induction ratio). Expression of MITF increased the relative luciferase activity about 5-fold in MeWo cells and more than 50-fold in HeLa cells transfected with pHTL12 containing the 3.6-kb 5′-flanking region of the human tyrosinase gene (44) (Fig. 8). Such a big difference in the magnitude of activation may be at least in part due to the fact that HeLa cells are deficient in MITF. Indeed, there were no significant differences between the normalized luciferase activities obtained with pSV2/L for HeLa and MeWo cells. The transactivation by MITF was seen only for the tyrosinase promoter and was not detectable for the heme oxygenase or SV40 early promoter. These results indicate that MITF specifically activates the tyrosinase gene promoter. In contrast, coexpression of a USF cDNA did notice-

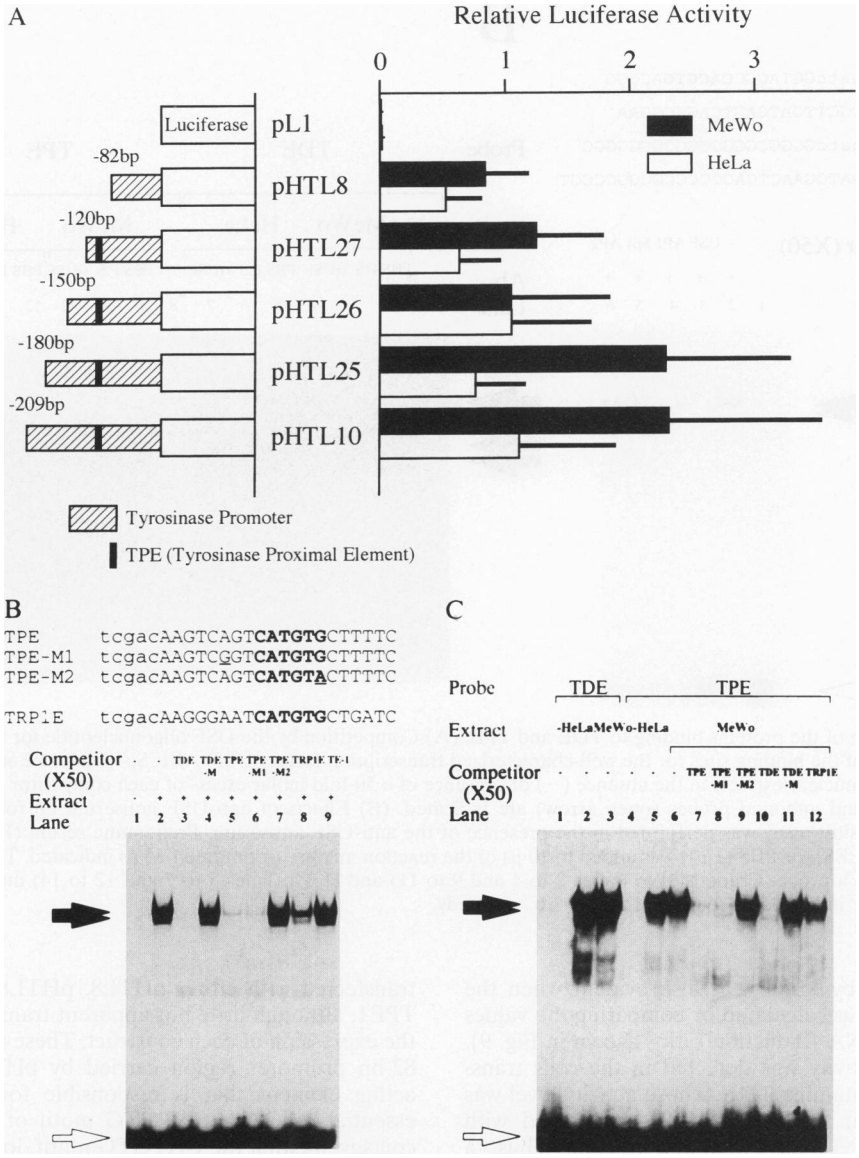

FIG. 5. Identification of the nuclear proteins that bind to TPE. (A) Functional analysis of the human tyrosinase promoter. The fusion plasmids, containing the deletion in the human tyrosinase promoter (positions are indicated in the figure) and the luciferase gene, are schematically shown on the left. The relative activity values shown on the right are means ± standard deviations (narrow bars) for four independent experiments. (B) Competition by TPE in the formation of the TDE-protein complexes. The sequences of the synthetic oligonucleotides TPE, TPE-M1, TPE-M2, and TRP1E are indicated at the top. TPE-M1 and TPE-M2 contain a single base substitution (underlined). A gel mobility shift assay was performed with TDE as a probe and MeWo nuclear extracts. Two major complexes are indicated by solid arrows, and unbound probes are indicated by an open arrow. A 50-fold molar excess of the cold competitor TDE, TDE-M, TPE, TPE-M1, TPE-M2, TRP1E, or TE-1 was added to lanes 3 to 9 as indicated. (C) Comparison of the binding activities interacting with TDE and TPE. TDE was used as a probe in lanes 1 to 3, and TPE was used in lanes 4 to 12. HeLa (lanes 2 and 5) and MeWo nuclear extracts (lanes 3 and 6 through 12) were used. The binding reaction was performed in the presence of a 50-fold molar excess of cold competitor TPE, TPE-M1, TPE-M2, TDE, TDE-M, or TRP1E (lanes 7 to 12).

ably increase the luciferase activities in both cell lines transfected with the human tyrosinase or heme oxygenase construct, but not with pSV2/L. The magnitude of activation of the tyrosinase and heme oxygenase promoters by USF was higher in HeLa cells than that in MeWo cells by more than 20-fold. It is noteworthy that the magnitude of activation of the tyrosinase promoter by MITF is slightly higher than that by USF in MeWo cells, but in HeLa cells USF transactivated the tyrosinase promoter more noticeably than did MITF. These results indicate that MITF is a factor required for the pigment cell-specific transcription of the tyrosinase gene and suggest that USF may be involved in the efficient transcription of the tyrosinase gene.

To localize the *cis*-acting element responsible for transactivation by MITF, we repeated coexpression assays with both HeLa and MeWo cells (Fig. 9). In this series of experiments, the relative luciferase activities were also calculated by comparing the values with those for pSV2/L. Coexpression of an MITF cDNA always induced relative luciferase activity in both cell lines transfected with any tyrosinase-luciferase constructs.

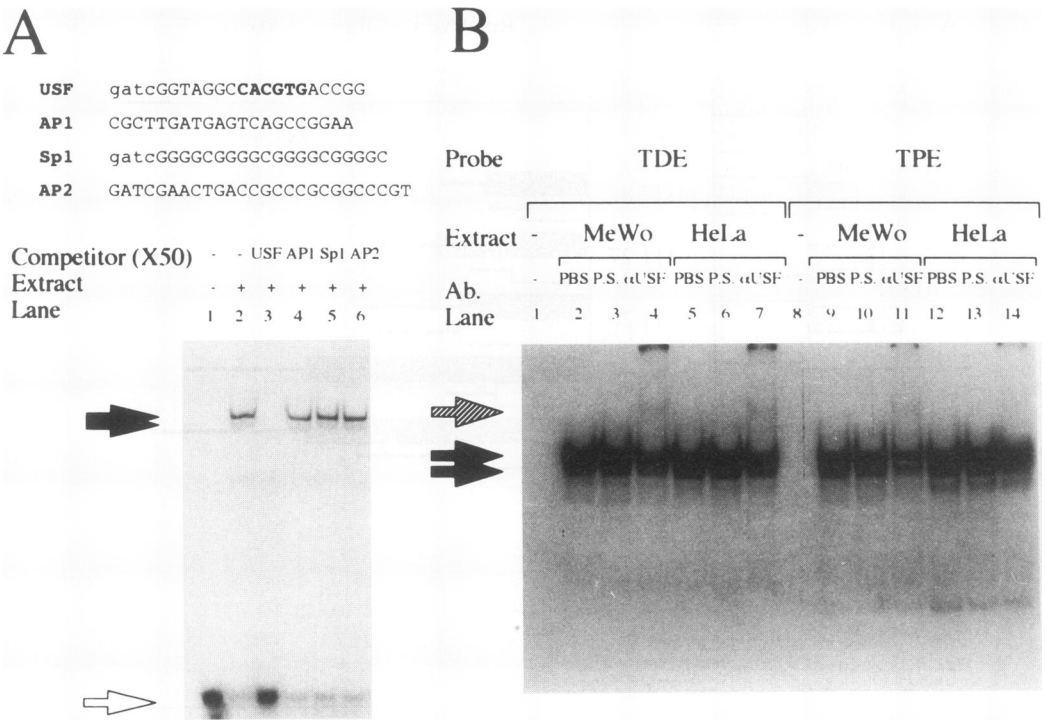

FIG. 6. Characterization of the proteins binding to TDE and TPE. (A) Competition by the USF oligonucleotide for the TDE-binding activity. The nucleotide sequences of the binding sites for the well-characterized transcription factors USF, AP1, Sp1, and AP2 are shown at the top. TDE was incubated with MeWo nuclear extracts in the absence (−) or presence of a 50-fold molar excess of each competitor (lanes 2 to 6). Two major complexes (solid arrows) and unbound probes (open arrow) are indicated. (B) Effects of anti-USF antiserum on formation of DNA-protein complexes. A gel mobility shift assay was performed in the presence of the anti-USF antiserum. Preimmune serum (P.S.), anti-USF antiserum (αUSF; diluted 1:100 with PBS), or PBS (1 μl) was added to 10 μl of the reaction mixture or omitted (−) as indicated. TDE (lanes 1 to 7) or TPE (lanes 8 to 14) was used as a probe. Crude MeWo (lanes 2 to 4 and 9 to 11) and HeLa (lanes 5 to 7 and 12 to 14) nuclear extracts were used. Supershifted complexes are indicated by a hatched arrow. Ab., antibody.

The marked activation by MITF was also evident when the magnitude of increase was calculated by comparing the values with those for vector DNA (induction ratios shown in Fig. 9). The highest level of activity was detected in the cells transfected with pHTL12 containing TDE, whose activity level was significantly higher than that in the cells transfected with pHTL6 containing the 1.8-kb 5′-flanking region. Thus, a principal MITF-responsive element is located in the region between −3.6 and −1.8 kb. The level of expression of either pHTL6 or pHTL10 containing TPE was similarly increased by MITF and was always higher than that of pHTL8 lacking TPE, suggesting that TPE is also responsible for MITF. It is noteworthy that the expression level of pHTL8 is also increased by MITF, although the magnitude of transactivation was low. All these results are essentially consistent for both cell lines except that the magnitude of activation is greater in HeLa cells than in MeWo cells, as seen in Fig. 8.

To examine whether TDE is responsible for the activation by MITF, we constructed fusion genes containing TDE or TDE-M at the 5′ end of the promoter region of pHTL8 (Fig. 10). Preliminary experiments confirmed that the insertion of one or three copies of TDE resulted in a 10-fold increase in the relative luciferase activity in MeWo cells but not in HeLa cells, while similar constructs with TDE-M did not affect promoter activity, even in MeWo cells (data not shown). Coexpression assays with HeLa cells showed that MITF increases the expression of the construct containing one or three copies of TDE by more than 50-fold (Fig. 10). In contrast, the relative levels of luciferase activity were similarly lower in the cells transfected with either pHTL8, pHTL8/TDEM3, or pHTL8/TPE1, although little but apparent transactivation was seen in the expression of each construct. These results suggest that the 82-bp promoter region carried by pHTL8 contains the *cis*-acting element that is responsible for MITF. Because the essential role of a CATGTG motif of TDE is evident, it is conceivable that the CATGTG motif, located at positions −12 to −7, is also involved in transactivation by MITF. Interestingly, the presence of two copies of TPE conferred a remarkable activation by MITF in the expression of pHTL8/TPE2. These results confirm the significance of the CATGTG motif of TDE and are consistent in part with our assumption that TPE is a weak enhancer for pigment cell-specific expression (Fig. 5 and 9). We therefore conclude that TDE is a principal *cis*-acting element responsible for transcriptional activation of the human tyrosinase gene by MITF.

DISCUSSION

We have identified the two *cis*-acting elements in the human tyrosinase gene, TDE and TPE, each of which contains the CATGTG motif in the center. TDE (positions −1861 to −1842) functions as a strong enhancer that directs pigment cell-specific expression, while TPE (positions −112 to −93) possibly functions as a weak pigment cell-specific enhancer. The fact that the CATGTG motif of each element is essential for specific protein binding and pigment cell-specific enhancer function led us to examine the possible involvement of USF or MITF containing a bHLH-LZ structure. Several transcription

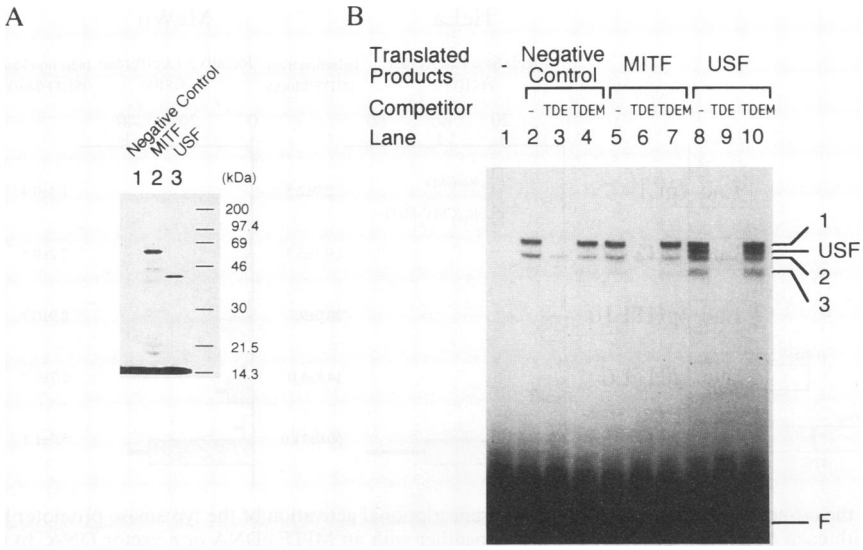

FIG. 7. Interaction between in vitro-translated USF and TDE. (A) Translation of MITF or USF mRNA, transcribed in vitro. The proteins labeled with [^{35}S]methionine were analyzed on a sodium dodecyl sulfate–10% polyacrylamide gel prior to autoradiography. In vitro translation was performed in the absence of exogenous RNA (lane 1) or in the presence of RNA (0.5 μg) for MITF (lane 2) or USF (lane 3). (B) Binding of USF to TDE. MITF or USF was synthesized by parallel in vitro translation in the presence of cold methionine instead of [^{35}S]methionine and was then subjected to a gel mobility shift assay with TDE as a probe. The binding reaction included each translation mixture containing either no exogenous RNA (lanes 2 to 4), MITF RNA (lanes 5 to 7), or USF RNA (lanes 8 to 10). A 50-fold molar excess of the cold competitor, TDE (lanes 3, 6, and 9) or TDEM (lanes 4, 7, and 10), was added as indicated. The two TDE-specific complexes (bands numbered 1 and 3 on the right side) and the one nonspecific complex (numbered 2) were possibly caused by the endogenous proteins in reticulocyte lysates. F, unbound probes.

factors such as TFEB, TFE3, and Myc family oncoproteins containing a bHLH-LZ structure have been reported and can bind specific sequences such as CACGTG or CATGTG (21). These proteins are well known to form dimers through a bHLH-LZ structure, and both USF and MITF are predicted to contain a bHLH-LZ structure (15, 16). We thus examined the effects of overexpression of USF or MITF on the human tyrosinase gene promoter. The results established that MITF specifically transactivates the tyrosinase promoter but not the heme oxygenase and SV40 early promoters (Fig. 8). In addition, MITF transactivated the tyrosinase gene promoter mainly through the CATGTG motif of TDE (Fig. 10). In contrast, USF did activate the promoter function of both the tyrosinase and heme oxygenase genes, indicating that this ubiquitous factor is not specific for the tyrosinase gene promoter. Therefore, we propose that MITF is a novel protein that is responsible for the melanocyte-specific transcription of the tyrosinase gene.

It is perplexing that the TDE- and TPE-binding activities were detected in HeLa cells, which do not express the tyrosi-

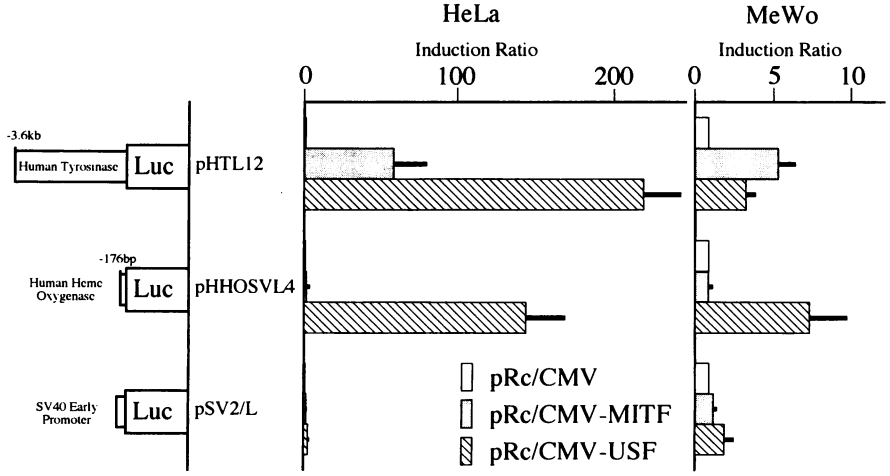

FIG. 8. Effects of coexpression of MITF or USF cDNA on the tyrosinase and heme oxygenase gene promoter activities. MeWo or HeLa cells were cotransfected with each promoter-luciferase construct (4 μg) and a test plasmid (4 μg; which includes pRc/CMV-MITF, pRc/CMV-USF, and a vector DNA). pHHOSVL4 contains the human heme oxygenase gene promoter upstream of the luciferase gene (Luc) (see Materials and Methods). The magnitude of activation is presented as the ratio of normalized luciferase activity obtained with a MITF or USF cDNA to that with vector DNA (induction ratio). The data shown are means ± standard deviations (narrow bars) for three independent experiments.

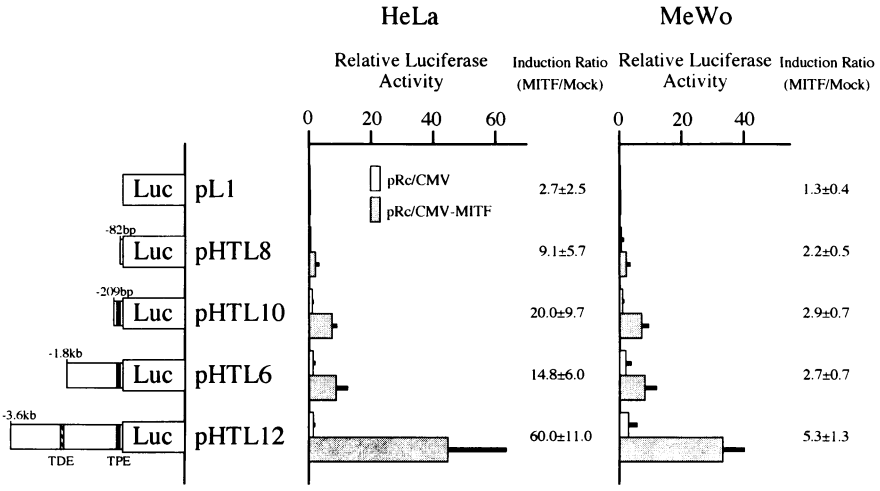

FIG. 9. Localization of the *cis*-acting elements responsible for transcriptional activation of the tyrosinase promoter by MITF. MeWo or HeLa cells were cotransfected with each tyrosinase-luciferase construct together with an MITF cDNA or a vector DNA. In this series of experiments, the normalized luciferase (Luc) activity, obtained by coexpressing each tyrosinase-luciferase construct and a test plasmid (MITF cDNA or vector DNA), was divided by the corresponding value obtained with pSV2/L and a test plasmid and is shown as the relative luciferase activity. The magnitude of activation is the ratio of normalized luciferase activity obtained with MITF cDNA to that with vector DNA (induction ratio). The data shown are means ± standard deviations (narrow bars) for four independent experiments.

nase gene, and were indistinguishable in gel mobility shift assays from those seen in melanoma cells (Fig. 4B). One possibility is that the TDE-binding protein(s) seen in melanoma cells is distinct from that seen in HeLa cells, although we were unable to detect the differences. Alternatively, the TDE-binding activities may be due to ubiquitous factors such as USF but the protein(s) associated with the TDE-binding proteins is expressed in a pigment cell-specific fashion and is responsible for cell-specific expression of the tyrosinase gene. Indeed, many properties of the TDE-binding proteins are consistent with those of USF, such as the elution profile in ion-exchange chromatography and the binding patterns in the gel mobility shift assay. In addition, the supershifted band was detected with the anti-USF antiserum (Fig. 6). We also confirmed by immunoblot analysis of the cotransfected HeLa and MeWo cells with the anti-USF antiserum that the immunoreactive USF is markedly increased in the nuclear extracts of cells expressing a USF cDNA but is not increased in extracts of cells expressing MITF cDNA, suggesting that the anti-USF antiserum does not cross-react with MITF (data not shown). It was then confirmed that USF is a component of the TDE-protein complexes by in vitro transcription and translation experiments, whereas we were unable to detect the DNA-binding activity of MITF (Fig. 7). This result suggests that certain posttranslational modifications may be required for the function of MITF or the conditions employed for the gel mobility shift assays are not suitable to detect the DNA-binding activity of MITF. Alternatively, MITF may form a dimer with a

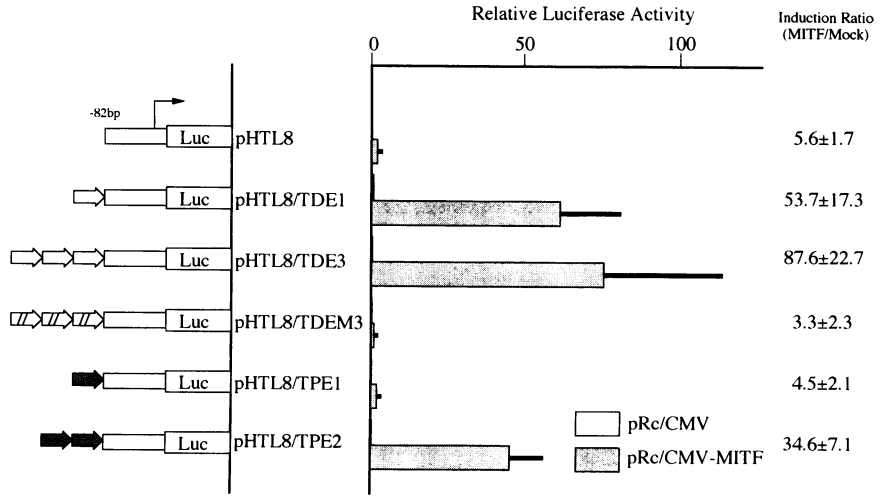

FIG. 10. Transcriptional activation of the tyrosinase promoter by MITF through TDE. HeLa cells were cotransfected with MITF cDNA and a construct containing either TDE (open arrows), TDE-M (diagonally striped arrows) or TPE (shaded arrows). Note that the relative luciferase (Luc) activities in vector-transfected cells are not clearly seen at this scale. The data shown are means ± standard deviations (narrow bars) for three independent experiments. Other conditions were the same as described in the legend for Fig. 9.

ubiquitous factor possessing a DNA-binding activity (for example, USF or TFE3), which in turn functions to direct the pigment cell-specific transcription of the tyrosinase gene.

The 270-bp upstream region of the mouse tyrosinase gene was shown to be sufficient to confer cell-specific expression in the transgenic mice (23) and in MeWo human melanoma cells (44). Within this 270-bp region, there are two copies of a CATGTG motif (positions −104 to −99 and −12 to −7), which may be crucial for its pigment cell-specific expression. The CATGTG motif is also found in both mouse and human TRP-1 gene promoters (41, 45). Lowings et al. identified the 11-bp *cis*-acting element containing the CATGTG motif of the mouse TRP-1 gene, termed the M box, that is required for a basal level of expression in B16 mouse melanoma cells (26). Recently, USF was shown to bind to the M box of the mouse TRP-1 gene (53). Interestingly, TPE contains the M box (positions −107 and −97) but its flanking sequences are different from those of the TRP-1 M box. The TDE sequence also deviates from the M-box sequence except for a CATGTG motif. In this context, we have shown that the promoter region of the human TRP-1 gene, containing TRP1E (Fig. 5B), is not able to direct its pigment cell-specific transcription (45). TRP1E contains a CATGTG motif but only slightly inhibits the formation of TDE- or TPE-protein complexes (Fig. 5B and C), suggesting that the sequence adjacent to a CATGTG motif is also required for efficient protein binding. Thus, it is of significance to investigate whether a common factor such as MITF is involved in the pigment cell-specific expression of the TRP-1 and TRP-2 genes.

A complete correlation between the protein-binding ability and pigment cell-specific enhancer function of TDE indicates the involvement of the TDE-binding proteins in pigment cell-specific expression of the tyrosinase gene. To identify the TDE-binding proteins found in melanoma cells, we are attempting to further purify the TDE-binding proteins. It is of significance to examine whether MITF forms dimers with USF or MITF binds to TDE. These studies will help us to understand the mechanism by which the tyrosinase gene is transcribed in a pigment cell-specific manner.

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